

Correlation of house dust mite-specific lymphocyte proliferation with IL-5 production, eosinophilia, and the severity of symptoms in infants with atopic dermatitis

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Because house dust mite (HDM)-specific IgE antibody (IgE-RAST) is usually not detectable in infants with atopic dermatitis (AD), HDM has not been regarded as the cause of infantile AD. The level of HDM-specific lymphocyte proliferation (expressed as stimulation index measured by flow cytometry [SIF]), however, was found to be markedly elevated in AD infants. This suggests that the sensitization of T cells to HDM extract occurs even in infancy. Moreover, the level of HDM-SIF is correlated closely with the severity of infantile AD, suggesting that HDM is a major cause of this disease not only in adults and children but also in infants. Although the level of HDM-SIF did not correlate with the level of HDM-specific IgE-RAST in infants with AD, it did intimately correlate with the absolute number of peripheral blood eosinophils. Because T lymphocytes are known to secrete some cytokines, such as IL-5, that enhance the proliferation of eosinophils, we measured the IL-5 production by peripheral blood mononuclear cells in infants with AD on stimulation with HDM extract. The amount of IL-5 production is significantly higher in infants with AD, as well as in children with AD, than that found in nonatopic control subjects. Moreover, the level of IL-5 production is correlated closely with the level of HDM-SIF in infants with AD. Taken together, these results suggest that HDMs play an important role in the development of infantile AD by inducing IL-5 production from HDM-specific T lymphocytes. (*J Allergy Clin Immunol* 1998;101:84-9.)

Key words: Atopic dermatitis, infants, house dust mite, lymphocyte proliferation, IL-5, eosinophils, flow cytometry

House dust mites (HDMs) are regarded as the major cause of atopic dermatitis (AD) in adults.^{1,2} Although the role is not yet clear, the serum level of HDM-specific IgE antibody (IgE-RAST) is usually high in those patients and used as a diagnostic criterion.¹ The prevalence of AD, however, is highest in infants.³ In contrast to adults, HDMs have not been regarded as the major allergen for infantile AD because their IgE-RAST level for HDM is usually very low or negative.⁴ Instead, food allergens, such as egg white, are thought to play an

Abbreviations used

AD:	Atopic dermatitis
HDM:	House dust mite
PBMC:	Peripheral blood mononuclear cells
PBS:	Phosphate-buffered saline
SIF:	Stimulation index measured by flow cytometry

essential role in the formation of infantile AD because the IgE-RAST levels for food allergens are elevated,⁴ and the severity of symptoms is often influenced by withdrawal or challenge of food allergens.^{1,3}

Recently, some investigators reported that the avoidance of HDMs in infancy suppresses the development of HDM-related airway allergic disorders in childhood.^{5,6} These findings suggest that sensitization to HDMs starts during infancy. Therefore it may be very useful to monitor sensitization to HDMs in infancy for the treatment as well as the prevention of HDM-related allergic disorders. Although there are so far no established methods for this purpose, it is well known that stimulation of peripheral blood mononuclear cells (PBMCs) with HDM extract in patients with AD causes the proliferation of HDM-specific helper T lymphocytes.⁷⁻⁹

In this study the usefulness of HDM-specific lymphocyte proliferation as an indicator of sensitization to HDMs in infancy was estimated. To clarify the function of HDM-specific T lymphocytes in infants with AD, we also measured the amount of IL-5 production by PBMCs on stimulation with HDM extract. In this article it is demonstrated that the HDM-specific lymphocyte proliferation, expressed as SIF (stimulation index measured by flow cytometry), is markedly elevated in infants with AD and intimately correlated with the severity of the disease, peripheral blood eosinophilia, and the amount of IL-5 production by PBMCs.

METHODS

Subjects

Heparinized peripheral blood was obtained at the time of routine blood examination from 56 patients with AD who visited our unit from December 1995 to March 1997 after informed consent was obtained. As controls, heparinized blood was collected similarly from 34 nonatopic children who did not

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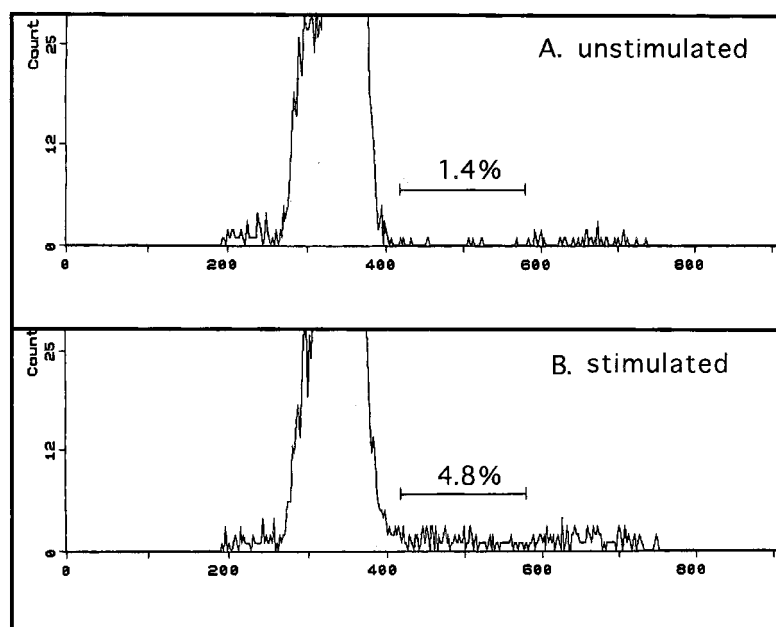


FIG. 1. Measurement of proliferating cells in PBMCs in a patient with AD. Percentage of S-phase cells in PBMC in patient with AD was measured after 6 days of culture with (B) or without (A) 50 μ g/ml of HDM allergen.

have any hematologic, immunologic, or infectious diseases. The median and range of the age of subjects were as follows: infants with AD, 6.5 months (range, 3 to 11 months) ($n = 18$); nonatopic infants, 5.5 months (range, 2 to 11 months) ($n = 12$); children with AD, 4.5 years (range, 1 to 15 years) ($n = 38$); and nonatopic children, 4 years (range, 1 to 15 years) ($n = 22$). Symptomatic AD was treated by topical use of steroid ointment, and none of the subjects were given steroids systemically. This study was approved by the Ethics Review Board of the Shizuoka Children's Hospital.

Estimation of the severity of AD

The severity of infantile AD is determined by the distribution of the severest skin lesions. This method was devised by modifying the methods described in some previous reports^{10,11} to enable an instant estimation in the outpatient office. To estimate distribution of lesions, the body is divided into three parts: the face (head), the extremities, and the trunk. The severest lesion on each part is selected and graded according to 6 degrees: none, slight, weak, medium, strong, and extraordinarily strong. Then the highest degree for each body part is chosen, and the number of parts showing this highest degree is counted. On the basis of the highest degree and the number of parts, the grade of the total severity of infantile AD is determined as follows: extremely severe = extraordinarily strong lesions on more than two body parts; severe = an extraordinarily strong lesion on one body part or strong lesions on more than two body parts; moderate = a strong lesion on one body part or medium lesions on more than two body parts; mild = a medium lesion on one body part or weak lesions on more than two body parts; minimum = a weak lesion on one body part or a slight lesion or lesions on at least one body part; and none = no lesions. For example, a patient with a strong lesion on one body part is classified as moderate, whereas a patient with strong lesions on two or three body parts is classified as severe irrespective of weaker lesions on other body parts. For statisti-

cal analysis, the severity score is allocated to each degree of the total severity of AD as follows: extremely severe = 5, severe = 4, moderate = 3, mild = 2, minimum = 1, and none = 0.

Cell culture

PBMCs were collected by gradient centrifugation over Ficoll-Paque (Pharmacia) and resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Xavier Investments, Wacol, Australia), 2 mmol/L glutamine, and antibiotics (100 U/ml penicillin and 100 μ g/ml of streptomycin). The cell suspension (0.5 ml) was distributed in each well in duplicate on a 48-well culture plate (Falcon 3078; Becton Dickinson), and cells were cultured for 6 days in a humidified atmosphere of 5% CO₂ at 37°C with or without 50 μ g/ml HDM (Der f) extract^{7,12,13} or 100 μ g/ml *Candida albicans* extract (Torii Pharmaceutical Co., Tokyo, Japan). The concentration of allergens and the duration of the culture were determined by preliminary experiments. After incubation, the supernatant was collected and stored at -80°C for measurement of IL-5. Cells were washed twice with phosphate-buffered saline (PBS) and fixed with 70% ethanol for analysis of HDM-specific lymphocyte proliferation.

Measurement of HDM-specific lymphocyte proliferation

In this study the antigen-specific lymphocyte proliferation was measured by a newly developed method in which no radioisotope is needed. Samples for measurement of lymphocyte proliferation were prepared in the same way as those for cell-cycle analysis.¹⁴ After washing twice with PBS, RNA was removed by incubating the fixed cells in 500 μ l PBS containing 100 μ g/ml RNase (Sigma) at 37°C for 1 hour. Cellular DNA was stained by adding propidium iodide (Sigma) at a final concentration of 40 μ g/ml. The amount of DNA per cell was

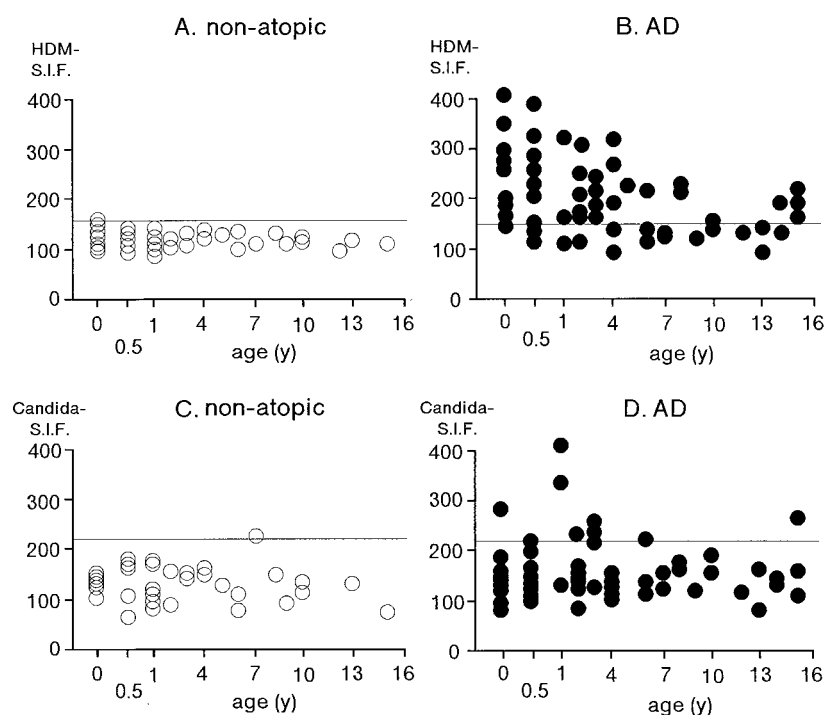


FIG. 2. Age-dependent changes of HDM- and *Candida*-SIF. The changes in levels of HDM- (A and B) and *Candida*-SIF (C and D) with age in subjects with AD (B and D) and nonatopic subjects (A and C) are shown. Horizontal lines in A and B indicate level of 155.3, mean + 2 SD of the HDM-SIF in all nonatopic subjects ($n = 34$), and those in C and D indicate level of 218.0, mean + 2 SD of the *Candida*-SIF in all nonatopic subjects ($n = 33$).

TABLE I. Level of HDM- and *Candida*-SIF in the children with AD

	Age (yr)	Subjects with AD (n)	Nonatopic subjects (n)	
HDM-SIF	0	248.4 ± 86.1 (18)	121.3 ± 21.8 (12)	$p < 0.001$
	1-2	201.3 ± 68.1 (10)	110.1 ± 20.6 (8)	$p < 0.01$
	3-15	175.4 ± 53.9 (28)	117.4 ± 14.0 (14)	$p < 0.001$
<i>Candida</i> -SIF	0	150.6 ± 49.1 (17)	147.8 ± 36.7 (11)	NS
	1-2	200.7 ± 95.5 (10)	133.5 ± 35.7 (8)	NS
	3-15	154.6 ± 46.9 (26)	130.9 ± 43.8 (14)	NS

Data are presented as mean ± 1SD.
NS, Not significant.

analyzed by flow cytometry (EPICS Elite, Coulter Corporation), and the percentage of the S-phase cells that appeared in the flat region between the peaks of the G0/G1- and G2/M-phase cells was measured (Fig. 1). The percentage of the

S-phase cells was converted to stimulation index by using the following equation and expressed as SIF.

$$\text{SIF} = \frac{\text{S-phase cell (\%)} \text{ in HDM-stimulated sample}}{\text{S-phase cell (\%)} \text{ in nonstimulated sample}} \times 100$$

Samples were prepared in duplicate and the mean was recorded as the SIF of each subject.

Measurement of IL-5 production, IgE-RAST, and eosinophils

IL-5 in the culture supernatant was measured with the ELISA kit (Quantikine human IL-5 immunoassay, R&D Systems). Its sensitivity is 1.0 pg/ml. HDM (Der f)-specific IgE-RAST was measured with a CAP-RAST radioimmunoassay kit (Pharmacia). The percentage of peripheral blood eosinophils was measured by an automated differential cell counter (Coulter MAXM, Coulter Corporation). The absolute number of peripheral blood eosinophils was calculated with the following equation:

$$\text{Absolute number of eosinophils} = (\text{percentage of eosinophils/100}) \times \text{absolute number of leukocytes.}$$

Statistics

SIF is expressed as the mean ± 1 SD, and IL-5 is expressed as the median and the range. The significance of the difference was determined by Student's *t* test for SIF, and the Wilcoxon rank sum test for IL-5. Correlation between the levels of SIF and IL-5 production is expressed by Spearman's rank correlation coefficient.

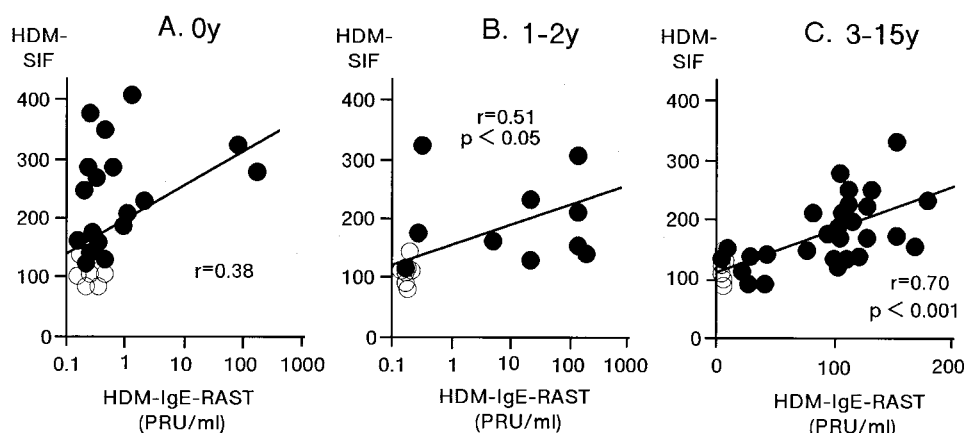


FIG. 3. Correlation between HDM-SIF and HDM-specific IgE-RAST in three age groups of 0 (A), 1 to 2 (B), and 3 to 15 years (C) is shown. Open circles indicate nonatopic subjects, and filled circles indicate subjects with AD.

TABLE II. IL-5 production by HDM-stimulated PBMCs

Age (yr)	N	Subjects with AD		Nonatopic subjects		
		N	IL-5 (pg/ml)	N	IL-5 (pg/ml)	
0	10		19.9 (1.0~169.0)	5	1.1 (<1.0~3.7)	$p < 0.05$
1-15	14		143.0 (14.8~438.2)	7	20.8 (<1.0~64.8)	$p < 0.05$

The amount of IL-5 is expressed as the mean and the range.

RESULTS

Elevated HDM-specific SIF level in infants with AD

There were no apparent age-dependent changes in the level of HDM-SIF in nonatopic subjects (Fig. 2, A). On the contrary, the level of HDM-SIF showed a remarkable age-dependent change in patients with AD. The HDM-SIF level was highest in infants and decreased gradually with age (Fig. 2, B). In contrast, the SIF level for *Candida albicans* extract did not show such an age-dependent change even in patients with AD (Fig. 2, C and D). The HDM-SIF levels were significantly higher in patients with AD than in the nonatopic control subjects in every age group, whereas there were no significant differences in the *Candida*-SIF levels between subjects with AD and control subjects (Table I).

There was a significant positive correlation between the levels of HDM-SIF and HDM-specific IgE-RAST in children with AD (Fig. 3, B and C) but not in infants with AD (Fig. 3, A).

Correlation of HDM-SIF with the severity of the disease and the absolute number of peripheral blood eosinophils in infants with AD

The level of HDM-SIF was found to be closely correlated with the severity of the disease in infants with

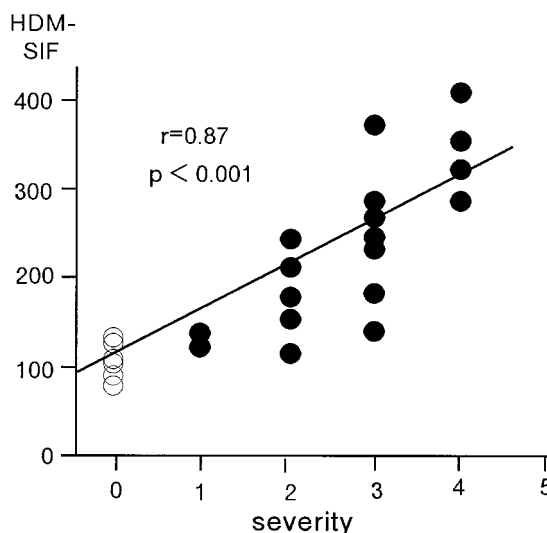


FIG. 4. Correlation between levels of HDM-SIF and severity of symptoms in infants with AD is shown. Open circles indicate nonatopic subjects, and closed circles indicate subjects with AD.

AD (Fig. 4). As was reported previously,^{15, 16} the severity of the disease in infants with AD was also correlated positively with the absolute number of peripheral blood eosinophils ($r = 0.78$, $p < 0.001$) (data not shown). Therefore the relationship between the level of HDM-SIF and the degree of eosinophilia was examined. There was a close positive correlation between the level of HDM-SIF and the absolute number of peripheral blood eosinophils in patients with AD (Fig. 5). The correlation coefficient between these factors was highest in infants (Fig. 5, A) and decreased with age (Fig. 5, B and C).

HDM-specific IL-5 production by PBMCs in infants with AD

IL-5 production was measured in 10 infants with AD (median age, 7 months; range, 3 to 11 months), 5

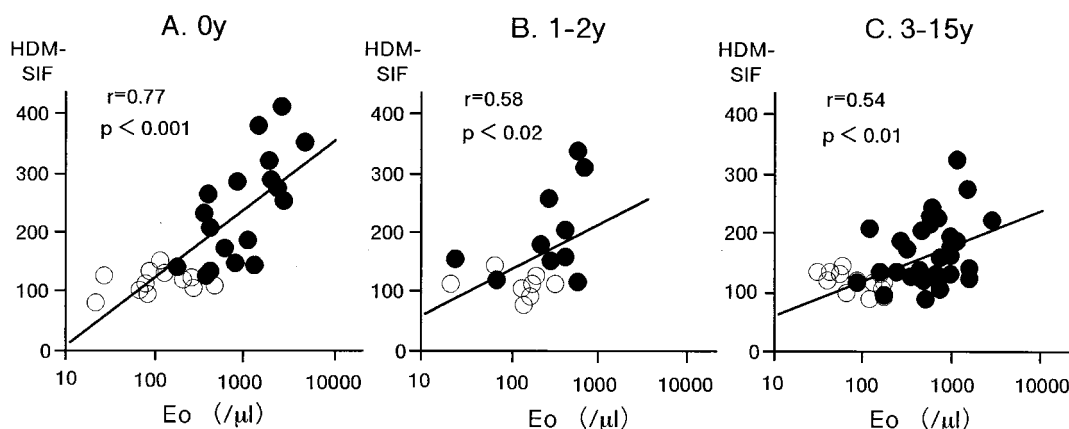


FIG. 5. Correlation between level of HDM-SIF and absolute number of peripheral blood eosinophils in three age groups of 0 (A), 1 to 2 (B) and 3 to 15 years (C) is shown. Open circles indicate nonatopic subjects, and closed circles indicate subjects with AD. Eo, Absolute number of eosinophils.

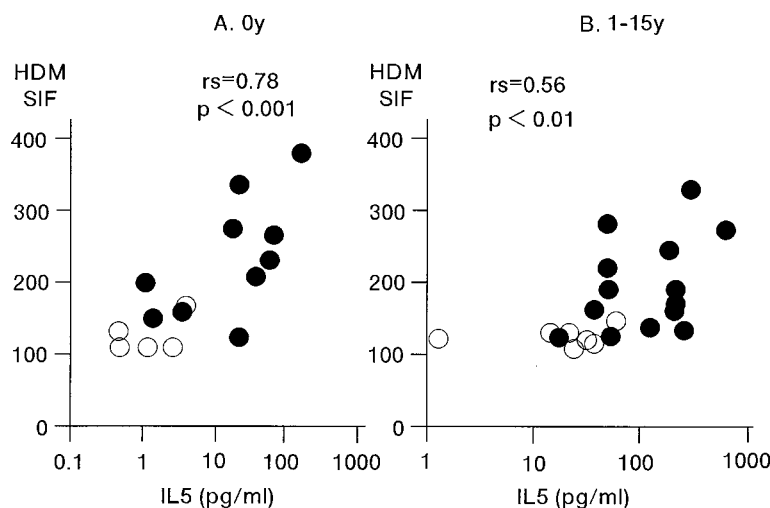


FIG. 6. Correlation between level of HDM-SIF and amount of IL-5 production by HDM-stimulated PBMCs in infants (0 years)(A) and children (1 to 15 years)(B) is shown. Open circles indicate nonatopic subjects, and closed circles indicate subjects with AD.

nonatopic infants (median age, 5 months; range, 2 to 11 months), 14 children with AD (median age, 6 years; range, 1 to 15 years), and 7 nonatopic children (median age, 8 years; range, 1 to 15 years). The amount of IL-5 production by HDM-specific lymphocytes in patients with AD was significantly higher than that in nonatopic controls not only in children (age, 1 to 15 years) but also in infants (age, 0 years) (Table II). The amount of IL-5 production correlated closely with the level of HDM-SIF in infants with AD, whereas there was only a weak positive correlation between these factors in children with AD (Fig. 6).

DISCUSSION

Although the level of HDM-specific IgE-RAST is usually very low in infants with AD, their HDM-specific lymphocyte proliferation was found to be markedly

elevated in this study. This may reflect the clonal expansion of HDM-specific helper T lymphocytes after sensitization. In children with AD, there was a significant positive correlation between the level of HDM-SIF and HDM-specific IgE-RAST, which agrees with previous reports.^{7,9} Because IgE synthesis is enhanced by IL-4 produced by T_{H2} cells,¹⁷⁻¹⁹ it could be assumed that HDM-specific T-cell clones not only expand but are also differentiated into T_{H2} cells in children with AD. In contrast, HDM-specific T lymphocytes in infants with AD do not appear to have the ability to upregulate the IgE synthesis because their HDM-specific IgE-RAST is usually negative despite the apparent elevation of the HDM-SIF level. Although the reason for the marked elevation of the HDM-SIF level in infants with AD remains to be elucidated, there may be an inverse relationship between the ability to proliferate and the

degree of the differentiation of HDM-specific helper T lymphocytes. This supposition seems to be consistent with the fact that the amount of IL-5 production by HDM-stimulated PBMCs is smaller in infants than in children (Table II).

The level of HDM-SIF, however, correlates closely with the severity of symptoms in infants with AD. This suggests that peripheral blood HDM-specific lymphocytes play an important role in the pathogenesis of infantile AD. So far, however, little is known about how HDM-specific T lymphocytes can participate in the development of infantile AD.

Eosinophils are now believed to play a critical role in allergic inflammation.²⁰ The number of eosinophils often increases in atopic patients and was reported to be correlated with the severity of AD in adults.^{15, 16} These findings are also observed in infants with AD. Moreover, it was revealed that there was a close positive correlation between HDM-SIF and the absolute number of peripheral blood eosinophils in infants with AD. Thus we hypothesize that peripheral blood HDM-specific T lymphocytes induce an increase in the number of eosinophils on stimulation with HDM by secreting cytokines such as IL-5, which enhances the proliferation of eosinophils.^{21, 22}

To confirm this hypothesis, we measured the IL-5 production by HDM-stimulated PBMCs in patients with AD. It was demonstrated that the level of IL-5 production by HDM-stimulated PBMCs was significantly higher not only in children with AD but also in infants with AD than in nonatopic control subjects. Thus it seems valid that one mechanism of peripheral blood HDM-specific T lymphocytes to participate in the development of infantile AD is the upregulation of the proliferation of eosinophils by secretion of IL-5. The level of IL-5 production per 1×10^6 cells is lower in infants with AD than in children with AD. However, because the number of peripheral blood T lymphocytes in infants is more than twice that found in children,²³ the difference in the actual amount of IL-5 production in vivo between infants with AD and children with AD should be smaller than that shown here.

The degree of the correlation between HDM-SIF and IL-5 production is apparently higher in infants with AD than in children with AD. To our knowledge, such a finding has not been reported. A close link between the proliferation and cytokine secretion seems to be a unique characteristic of infantile HDM-specific T lymphocytes. This may be the reason for the correlation between the level of HDM-SIF and the absolute number of eosinophils to be closer in infants with AD than in children with AD.

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